Universidade de Aveiro Departamento de Biologia
2012

CELSO FILIPE FERREIRA MARTINS

POLÍMEROS FUNCIONAIS COMO AGENTES ANTIMICROBIANOS PARA DESINFEÇÃO ORAL

FUNCTIONAL POLYMERS AS ANTIMICROBIAL AGENTS FOR ORAL DISINFECTION

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – Ramo de Microbiologia Clínica e Ambiental, realizada sob a orientação científica da Professora Doutora Maria Guilhermina Martins Moutinho, Professora Associada do Instituto Superior de Ciências da Saúde Egas Moniz, e coorientação da Professora Doutora Maria Ângela Sousa Dias Alves Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

Ao meu Pai, mentor e exemplo de vida...

agradecimentos

Muitas foram as pessoas que, de alguma forma contribuíram para que eu tivesse condições para a realização deste trabalho. Assim, antes de referir alguém em particular, um muito obrigado a todas.

No entanto não posso deixar de referir a minha orientadora de projeto de investigação, a Professora Doutora Guilhermina Moutinho, pela disponibilidade e simpatia, sendo incansável a todos os níveis, e a minha coorientadora de dissertação, a Professora Doutora Ângela Cunha, pela disponibilidade demonstrada em ajudar e levar a bom porto este trabalho.

À Sandra e à Susana, pela ajuda preciosa, companheirismo, alegria inigualáveis e um profissionalismo que não está ao alcance de todos.

Devo também deixar uma palavra de agradecimento à minha família, pelo carinho, apoio e compreensão. A todos os meus amigos por serem isso mesmo, amigos…pela presença, apoio e por saberem arrancar de mim sorrisos em ocasiões menos boas, com um timing e graciosidade apenas dignos de amizade sincera.

Aos meus amigos e irmãos da Tuna Universitária de Aveiro, um enorme muito obrigado por serem a minha segunda família. Repetindo a dedicatória acima mencionada ao meu pai, mentor e exemplo de vida.

Trabalho financiado, realizado no âmbito do projeto de investigação PTDC/QUI/73939/2006 “ACTIVPOL – Desenvolvimento de poli-oxazolinas e poli-aziridinas com atividade antimicrobiana utilizando tecnologia supercrítica”

o júri

Presidente	Professora Doutora Maria Adelaide de Pinho Almeida (Presidente) Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro
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palavras-chave Microflora oral, polímeros, poli-oxazolinas, MIC, desinfeção, higiene oral.

resumo

A flora microbiana da boca é uma das mais ricas e diversas do corpo humano. É no entanto, altamente variável de indivíduo para indivíduo, sendo por isso, impossível estabelecer com rigor o número e tipo de espécies que fazem parte desse bioma. Microrganismos patogénicos presentes na boca têm vindo a ser progressivamente associados a patologias de sistemas orgânicos que ultrapassam o âmbito estrito da cavidade oral. São diversos os casos descritos de endocardites, cancro, infeções respiratórias e até diabetes, provocados por microrganismos presentes na flora oral.

Dada a relevância clínica que as infeções orais têm vindo a assumir nos últimos anos, o conhecimento detalhado sobre a composição da microflora oral e a sua suscetibilidade a compostos antimicrobianos passíveis de aplicação em desinfeção oral reveste-se de particular relevância.

Foram efetuadas recolhas de amostras microbiológicas da boca de pacientes da clínica de medicina dentária universitária do Instituto Superior de Ciências da Saúde Egas Moniz, com o objetivo de isolar o maior número possível de estirpes encontradas para, posteriormente, se testar a eficácia antimicrobiana de dois oligómeros previamente sintetizados no decurso do projeto.

Foram isoladas 103 estirpes, representando 37 espécies diferentes. Cada um dos 103 isolados obtidos foi caracterizado quanto à sua suscetibilidade aos compostos PMETOX-DDA e LPEI através da determinação da concentração mínima inibitória (MIC). Os valores médios de MIC obtidos foram de 0,530 $mg.mL^{-1}$ para LPEI e 0,723 $mg.mL^{-1}$ para PMETOX-DDA, sendo os valores de dispersão mais elevados para LPEI. Ambos os polímeros foram eficazes e, possivelmente, complementares enquanto agentes antimicrobianos. Os resultados deste estudo são consistentes com os previamente obtidos em ensaios microbiológicos preliminares, confirmando as poli-oxazolinas como uma promissora nova alternativa terapêutica em alternativa a agentes antimicrobianos convencionais.

Paralelamente, informação sobre a composição da microflora dos diferentes compartimentos da boca foi analisada em conjunto com informação sobre a história clínica e hábitos de higiene oral de cada paciente, recolhidos por médicos dentistas através de observação e inquérito.

Foram obtidos resultados significativos do teste não-paramétrico de Kruskal-Wallis ($p < 0,05$) na análise a alguns hábitos de higiene e do historial clínico. Os valores da análise de similaridade e dissimilaridade entre grupos (SIMPER) foram estatisticamente relevantes, assim como a análise ANOSIM (global R = 0,765, com um nível de significância estatística de amostra de 0,1%) confirmando perfis de distribuição diferentes de acordo com as diferentes zonas da boca. A escovagem da língua e uso do fio dental são os hábitos de higiene oral mais relevantes na colonização oral por microrganismos.

keywords oral microflora, polymers, poly-oxazolines, MIC, disinfection, oral hygiene.

abstract

The microbial flora of the mouth is one of the richest and most diverse in the human body. However, it is highly variable from individual to individual being, therefore, impossible to determine the exact number and kind of species that are part of this biome.

Pathogens present in the mouth have been progressively associated with diseases of organic systems that go beyond the strict scope of the oral cavity. There are several reported cases of endocarditis, cancer, respiratory infections and even diabetes, caused by microorganisms in the oral flora.

Given the clinical relevance that oral infections have been taking in recent years, a detailed knowledge about the composition of oral microflora and its susceptibility to antimicrobial compounds likely to be applied in oral disinfection, assumes particular relevance.

Microbiological sampling of patients from the mouth of the university dental clinic of the Institute of Health Sciences Egas Moniz was conducted, in order to isolate the largest possible number of strains to subsequently test the efficacy of two antimicrobial oligomers, previously synthesized in the course of the project.

A set of 103 strains, representing 37 different species, was obtained. Each of the 103 isolates was characterized for their susceptibility to compounds PMETOX-DDA and LPEI by determining the minimum inhibitory concentration (MIC). Mean obtained MIC values were 0.530 $mg.mL^{-1}$ to LPEI and 0.723 $mg.mL^{-1}$ to PMETOX-DDA, being dispersion values higher to LPEI. Both polymers were effective and possibly complementary as antimicrobial agents. The results of this study are consistent with results previously obtained in preliminary microbiological assays, confirming poly(oxazoline)s as a promising new therapeutic alternative to conventional antimicrobial agents.

In addition, information on the composition of the microflora of the mouth of different compartments was analyzed along with information on the clinical history and oral hygiene habits of each patient, collected by dentists through observation and investigation.

Statistically significant results of non-parametric Kruskal-Wallis test were obtained ($p\text{-value}<0.05$) for some hygiene habits and medical history events. SIMPER analysis values of dissimilarity between groups and similarity within groups were statistically relevant, as well as the ANOSIM analysis (global $R=0.765$, with a significance level of sample statistic of 0.1%), confirming the existence of different distribution profiles according to different areas of the mouth. Tongue brushing and flossing are the more relevant oral hygiene habits in oral colonization by microorganisms.

CONTENTS

OUTLINE

The presented dissertation was performed in the framework of the research project PTDC/QUI/73939/2006 "ActivPOL - Development of oxazoline and aziridine-based antimicrobial polymers using supercritical carbon dioxide technology". The main purpose of this work was the evaluation of the efficiency of the previously synthesized polymers in a clinical context; however alongside the sampling, isolation, identification of the strains and the susceptibility tests, dental clinic diagnosis and hygiene habits data have been collected from the patients of the dental clinic of the Instituto Superior de Ciências da Saúde Egas Moniz that were part of this study. This study followed the requirements of the ethic committee of the institution, and all the patients were carefully informed about the study and signed an informed consent. All the clinical procedures were performed by medical dentists and dentistry students in curricular internship.

This dissertation is divided in two chapters, in order to provide a better understanding of the two different components of the study. The first chapter refers to the study about the ecology and distribution of microbial communities in the human mouth, and the possible role of the clinical history and hygiene habits in these events, whereas the second chapter presents the susceptibility tests of the studied oligomers.

Both chapters were based on the submitted papers that resulted from this work, having few text modifications plus the insertion of some figures and references, not used in the papers due to journals limitations. The submitted manuscript drafts are available in the appendix section of this thesis.

STATE-OF-ART

Oral infections have been receiving increasing attention in the past few years for their association with numerous systemic pathologies [1-4]. It is now accepted that oral health has direct influence in the global health of human being [2]. Several reports of cancer [4], endocarditis [5-8] and diabetes [3] have been related to inflammations driven by infections in oral cavity.

Advanced periodontitis, and periodontal region infections were scientifically proven to have direct relation to some cardiac and respiratory pathologies, namely endocarditis [1, 3, 5-10], however the oral microbial community is widely spread in various regions of the mouth [2, 10-16]. In this context the knowledge of the ecology and distribution of oral communities in the mouth may have an important role in prevention and treatment of oral infections. By knowing the typical communities present on the different regions of the oral cavity, it is possible to prevent and treat oral infections, as well as their associated systemic pathologies. Along with the distribution of the oral communities, it is also important to realize which of the microorganisms are potentially pathogenic by extensive literature reading, and by consulting case reports [6-8, 14, 15, 17-34]. Being the mouth and important pathway of entrance to microorganisms, due to its singular and favorable characteristics (temperature, humidity, mucous surfaces and high levels of oxygen), several microbial species (not indigenous of the mouth) establish there not causing major complications. However, these microorganisms often represent serious danger for human health, especially when associated with other clinical conditions, taking advantage of physical injuries by causing opportunistic infections and spreading by different organic systems of the body.

Conventional systemic antibiotics used in dentistry therapy, especially in the treatment of periodontal infections are mainly metronidazole-amoxicillin and metronidazole-ciprofloxacin based, suffering from several potential problems, including insufficient spectrum of antimicrobial activity in some periodontal infections and risks of producing an antibiotic resistant microbiota [35, 36]. There are also topical antibiotics used in oral infections therapy, nevertheless the development of resistant bacteria [36] associated to the dubious effectiveness of antimicrobial action and potential problems with selectivity of these antibiotics makes them constitute a less desirable choice than the preventive application of topical antiseptics [35]. However topical antiseptics are potentially toxic to both infectious agents and host cells, being their application in humans limited to infected wounds, skin and mucosa [35, 36]. Other specific examples of antimicrobials have been explored, such as povidone-iodine, which presents high antiseptic potential but may induce hyperthyroidism constituting a high risk to the patients, or chlorhexidine which is active mainly against bacteria but exhibits little bactericidal activity at low concentrations against enteric gram-negative rods, have been reported adverse reactions at higher concentrations [35].

Considering the disadvantages of the above described antimicrobials in terms of toxicity and development of resistance, being the last a crescent motive for concern [36], the development of new strategies of combat against microorganisms stands as an important topic of interest.

Several alternative antimicrobials have been studied recently [35, 37, 38], such as antimicrobial lipids [39]. However antimicrobial lipids and essential oils demonstrate an enormous potential in their action as food antimicrobials, reducing the risk of contamination by foodborne pathogens in the kitchen, and in food-preparing and food-processing facilities [39].

Therefore antimicrobial polymers have been synthesized and studied in the past few years, being the latest prospect of alternative to conventional antibiotics [40-45]. The accepted mechanism of action of these polymers is analogous to that of antimicrobial peptides [40, 41, 43] that occur naturally in human body, as constituents of our immune system [46-48]. The antimicrobial polymers show many characteristics, such as chemical stability, reduced residual toxicity, nonvolatile properties, restricted permeation through the skin and versatility of effects as biocidal agents which, combined to their accepted mechanism of action that hardly allows the development of microbial resistance [40, 41], makes them a promising alternative to conventional antibiotics when comparing these characteristics with those of above described conventional antimicrobials in terms of effectiveness, low risk of resistance development and low toxicity (unpublished data). Furthermore, these polymers may be used as systemic antimicrobials or as topical antimicrobials, permitting therapeutic and preventive applications, due to their reduced toxicity and restricted permeation through the skin.

The lack of clinical studies and clinical data about this class of antimicrobial compounds calls for studies that may validate it as an effective alternative to conventional antibiotics.

For all of this, the discovering of new antimicrobial compounds, associated to the deep knowledge of the dynamics and distribution of oral communities in the mouth, is considered a priority in terms of public health [36, 49].

Following the above mentioned research project and the performed primary microbiological analysis, this study appears as the first performed in a clinical context, using wild strains collected from real patients.

CHAPTER 1 – RELATIONS BETWEEN ORAL HYGIENE, MEDICAL HISTORY AND COMPOSITION OF MICROBIAL COMMUNITIES OF THE HUMAN MOUTH

OVERVIEW

In parallel with the collection of microbiological samples for isolation and identification of microorganisms from different micro-niches of the human mouth, data on the clinical history and hygiene habits of the patients were obtained by medical dentists. The ecology of oral microbial communities is still an underexplored topic. The objective of the work reported on this chapter was the establishment of patterns of occurrence and distribution of microorganisms in relation to clinical history and hygiene habits by means of a broad statistical analysis of microbiological and clinical data.

INTRODUCTION

The role of oral infections in human health has received increasing attention in the past few years. The mouth provides a favorable environment for the development of bacterial communities due to its humidity and temperature [1]. Presently, it is generally accepted that a good oral hygiene is an important factor, not only in the prevention of oral diseases, such as caries, periodontal disease or halitosis [2-7], but also of other major complications. Cancer, diabetes, endocarditis and respiratory diseases are some of the pathologies that have been directly related to pathogens involved in oral infections [7-10].

Streptococcus mutans, *Streptococcus oralis*, *Prevotella oralis*, *Prevotella melaninogenica* and *Actinomyces naeslundii* are some of the most frequent oral pathogens, associated to oral infections, periodontitis and other oral diseases. Several other microorganisms, such as *Enterococci*, *Gemella morbillorum*, *Streptococcus sanguinis*, *Streptococcus pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* are also commonly found in the mouth, and sometimes pointed as causative agents of other systemic and organic infections [2-7].

The importance of relating different parameters such as oral hygiene, food habits and clinical history with the incidence of the number of isolates and pathogens, figures as extremely important in terms of prophylaxis and public health. For instance, 5-20% of the population is estimated to be affected by advanced forms of periodontitis, which in turn, has been associated to the occurrence of respiratory infections, cardiovascular disease and diabetes. It is estimated that 15-20% of human tumors are caused by infection-driven inflammations, being the mouth an important pathway of transmission [7-10].

Tracing incidence profiles according to the different zones of the mouth may inform on the significant parameters of distribution of microorganisms and to identify the most sensitive compartments of the oral cavity.

Envisaging these objectives, we used culture-dependent methods of isolation and phenotypic characterization of microorganisms, by means of selective media, staining, microscopy and biochemical tests to achieve species identification. An innovative statistical approach was applied in order to enlighten significant relations between medical history and oral hygiene habits and the distribution of microorganisms within different compartments of the oral cavity.

MATERIALS AND METHODS

Isolation and characterization of clinical strains

Samples were collected from the oral cavity of 21 patients (18 to 65 years old) of the Dental Clinic of 'Instituto Superior de Ciências da Saúde Egas Moniz' (Portugal), in compliance of the instructions of the institutional ethic committee. Briefly, 500 µl of saliva was diluted in 9.5 mL of API suspension medium. Swabs of the cheek, teeth, palate, tongue and endodontic absorbent paper points were suspended in 10 mL of API suspension medium. The suspensions were diluted up to 10^{-3} concentration and inoculated in selective media.

Mitis Salivarius Agar (MSA) (BD-Difco), Mitis Salivarius Bacitracin (MSB) (BD-Difco), Chapman (bioMérieux), D-Coccosel (bioMérieux), Man Rogosa and Sharpe (MRS) (BD-Difco), Drigalsky (bioMérieux), Blood Agar (bioMérieux), CHROMagar Candida (BD-BBL) and Anaerobic Blood Agar (CDC) (bioMérieux) were used as selective media. Selective cultures were incubated according the microorganisms requirements with atmosphere generating systems of anaerobiose (AnaeroGen-Oxoid), CO_2 (CO_2Gen-Oxoid) and microaerophilia (CampyGen-Oxoid) during 24h, 48h or 72h. Characteristic and isolated colonies were selected for further purification.

FIGURE 1 – DISPLAY OF CULTURE MEDIA USED TO INOCULATE SAMPLES FROM A PATIENT.

Identification of clinical isolates

Pure cultures were identified after Gram staining, catalase and coagulase tests, with the API systems (API 20 Strep, API Staph, API 20E, API 50 CHL and API 20C AUX) (bioMérieux). The API systems were incubated according manufacture instructions (Figures 4 and 5). All the isolates were conserved in duplicate using Cryobank vials system and stored at -80°C [2-5].

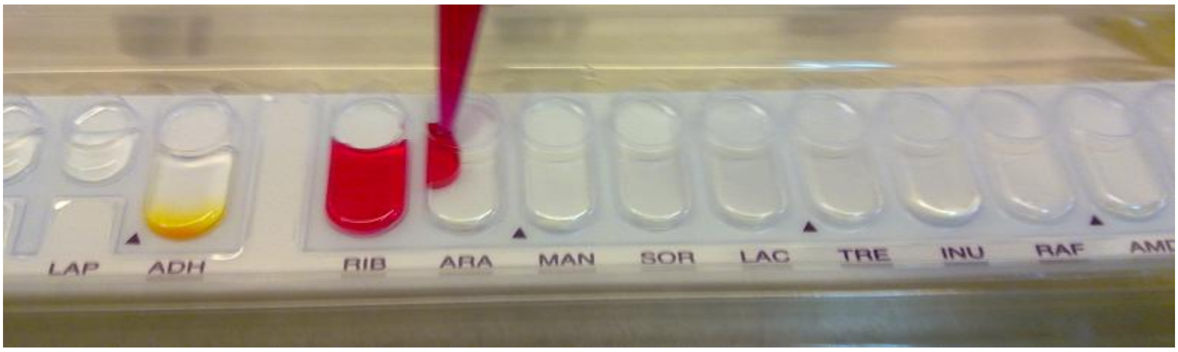

FIGURE 2 – INOCULATION OF API GALERIES.

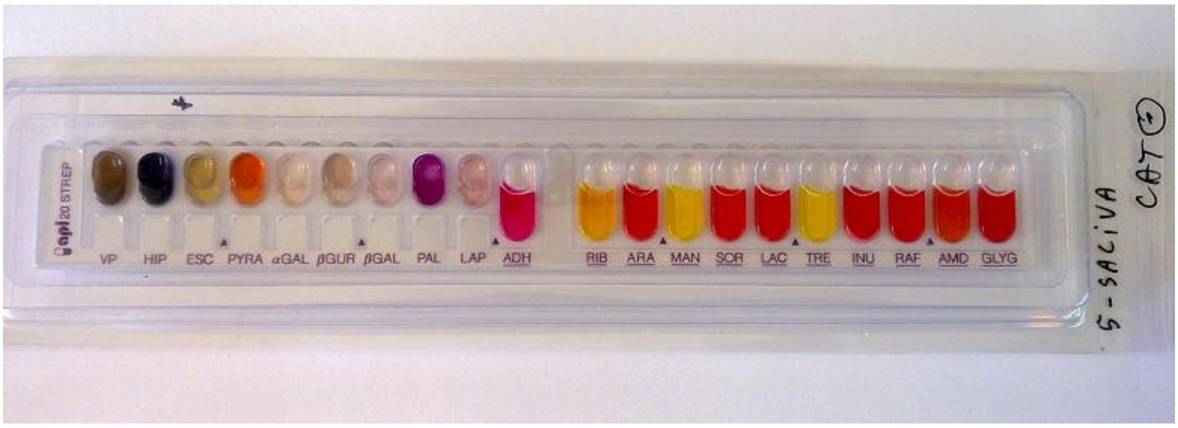

FIGURE 3 – EXAMPLE OF AN API 20 STREP TEST AFTER INCUBATION AND REACTION WITH THE REACTORS, READY FOR READING.

The organisms that were considered as pathogens were the ones with recurrent reports of infections, both in the oral and other systemic diseases, as commensals or as pathogens [11-33].

Assessment of clinical history and hygiene habits data.

Anamnesis and clinical observation were performed by medical dentists and dentistry students. Information on the hygiene habits of the patient was obtained through an inquiry (Table 1).

TABLE 1 – EVENTS COLLECTED IN THE SURVEY AND CLINICAL DATA ASSESSED BY DENTISTS ANALYSIS.

Inquiry items	
Clinical history	**Hygiene habits**
Dental prosthesis	Onycophagia
Braces	Carefull diet
Missing teeth	Tongue brushing
Gingival bleeding	Smoking habits
Teeth mobility	Alcohol
Treated caries	Mouth washers
Non-treated caries	Dental flossing

Statistical analysis

Data were analyzed with XL-STAT software (Microsoft) to obtain contingency tables and evaluate dependence coefficient values using non-parametric Kruskal-Wallis test (α=0,05), relating 14 clinical history events and hygiene habits (inquiry items presented in Table 1) identified from the survey, with the number of isolates and the number of pathogens isolated. In order to assess the distribution of the species and genus in the 6 different zones of the mouth the contingency tables were obtained using XL-STAT, and then transformed and analyzed using PRIMER 6 (Primer-E). Primary data transformation were performed using square root, log(x+1), and presence/absence methods. Square root transformation was the most adequate to the data set. Similarity Percentage (SIMPER) was obtained from transformed tables. Resemblance matrices were obtained from transformed tables using Bray-Curtis similarity. Multidimensional Scaling (MDS) and Analysis of Similarities (ANOSIM) were performed from the previously obtained resemblance matrices [34]. In addition to the 6 different mouth zones used as label factors, the label general was attributed to species occurring in similar abundance in more than one different zone. Therefore, general group was included in the statistical analysis of the distribution.

RESULTS

Isolation and identification of oral microbes.

The set of 103 isolates, representing 37 species was isolated from 6 different mouth zones. The most frequent species were Gram positive *Streptococcus salivarius* (n=13), *Aerococcus urinae* (n=10), *Staphylococcus aureus* (n=9) and *Streptococcus mitis* (n=9). Saliva was the biological product from which more isolates were obtained (n=28) (Table 2).

TABLE 2 – CHARACTERIZATION OF MICROBIAL ISOLATES IN TERMS OF ORAL COMPARTMENT AND PATHOGENICITY.

Patient	Mouth Zone	Species	Pathogenicity
1	Palate	*Streptococcus mitis*	Commensal
	Cheek	*Streptococcus mitis*	Commensal
2	Palate	*Streptococcus intermedius*	Pathogen
	Teeth	*Streptococcus intermedius*	Pathogen
	Saliva	*Staphylococcus lentus*	Pathogen
	Saliva	*Candida albicans*	Pathogen
3	Tongue	*Streptococcus uberis*	Commensal
	Teeth	*Streptococcus sanguinis*	Pathogen
	Teeth	*Aerococcus urinae*	Pathogen
	Teeth	*Staphylococcus xylosus*	Commensal
4	Palate	*Streptococcus bovis*	Pathogen
	Saliva	*Streptococcus salivarius*	Commensal
5	Palate	*Streptococcus salivarius*	Commensal
	Teeth	*Kocuria kristinae*	Commensal
	Teeth	*Lactococcus lactis spp.cremoris*	Commensal
	Cheek	*Aerococcus urinae*	Pathogen
	Cheek	*Streptococcus salivarius*	Commensal
	Saliva	*Streptococcus mitis*	Commensal
	Saliva	*Staphylococcus aureus*	Pathogen
	Saliva	*Staphylococcus xylosus*	Commensal
	Saliva	*Escherichia coli*	Pathogen
6	Teeth	*Kocuria kristinae*	Commensal
	Saliva	*Staphylococcus aureus*	Pathogen
	Saliva	*Candida albicans*	Pathogen
	Saliva	*Lactobacillus rhamnosus*	Commensal
7	Tongue	*Lactobacillus salivarius*	Commensal
	Teeth	*Candida albicans*	Pathogen
	Saliva	*Staphylococcus capitis*	Commensal
	Saliva	*Streptococcus salivarius*	Commensal
	Saliva	*Aerococcus urinae*	Pathogen
	Saliva	*Lactobacillus rhamnosus*	Commensal
8	Tongue	*Streptococcus mitis*	Commensal
9	Tongue	*Staphylococcus saprophyticus*	Pathogen
	Teeth	*Streptococcus mitis*	Commensal
	Teeth	*Aerococcus urinae*	Pathogen
	Teeth	*Streptococcus sanguinis*	Pathogen
	Periodontal	*Prevotella melaninogenica*	Pathogen
	Saliva	*Staphylococcus capitis*	Commensal
10	Saliva	*Streptococcus sanguinis*	Pathogen
11	Periodontal	*Staphylococcus epidermidis*	Pathogen
	Saliva	*Aerococcus urinae*	Pathogen
12	Teeth	*Staphylococcus aureus*	Pathogen
13	Periodontal	*Gemella morbillorum*	Pathogen
14	Palate	*Streptococcus salivarius*	Commensal

	Saliva	*Candida albicans*	Pathogen
15	Tongue	*Streptococcus salivarius*	Commensal
	Teeth	*Streptococcus salivarius*	Commensal
	Teeth	*Staphylococcus aureus*	Pathogen
	Periodontal	*Prevotella oralis*	Pathogen
	Cheek	*Streptococcus mutans*	Pathogen
	Saliva	*Staphylococcus aureus*	Pathogen
	Saliva	*Candida albicans*	Pathogen
16	Tongue	*Gemella morbillorum*	Pathogen
	Tongue	*Lactobacillus fermentum*	Commensal
	Cheek	*Streptococcus anginosus*	Pathogen
	Cheek	*Kocuria kristinae*	Commensal
	Saliva	*Staphylococcus aureus*	Pathogen
17	Tongue	*Aerococcus urinae*	Pathogen
	Tongue	*Staphylococcus epidermidis*	Pathogen
	Palate	*Streptococcus suis*	Pathogen
	Palate	*Streptococcus salivarius*	Commensal
	Teeth	*Streptococcus salivarius*	Commensal
	Teeth	*Staphylococcus aureus*	Pathogen
	Cheek	*Streptococcus mitis*	Commensal
	Cheek	*Streptococcus pneumoniae*	Pathogen
	Saliva	*Lactobacillus buchneri*	Commensal
18	Tongue	*Streptococcus suis*	Pathogen
	Palate	*Streptococcus salivarius*	Commensal
	Teeth	*Kocuria kristinae*	Commensal
	Teeth	*Aerococcus urinae*	Pathogen
	Cheek	*Streptococcus bovis*	Pathogen
	Saliva	*Lactobacillus fermentum*	Commensal
19	Tongue	*Enterococcus faecium*	Pathogen
	Tongue	*Staphylococcus chromogenes*	Pathogen
	Tongue	*Staphylococcus aureus*	Pathogen
	Tongue	*Enterococcus faecalis*	Pathogen
	Palate	*Streptococcus mitis*	Commensal
	Palate	*Leuconostoc spp.*	Pathogen
	Palate	*Streptococcus mutans*	Pathogen
	Palate	*Enterococcus avium*	Pathogen
	Palate	*Staphylococcus epidermidis*	Pathogen
	Teeth	*Streptococcus oralis*	Pathogen
	Periodontal	*Actinomyces naeslundii*	Pathogen
	Cheek	*Aerococcus urinae*	Pathogen
	Cheek	*Streptococcus mitis*	Commensal
	Cheek	*Streptococcus termophilus*	Pathogen
	Saliva	*Streptococcus mutans*	Pathogen
	Saliva	*Staphylococcus aureus*	Pathogen
	Saliva	*Candida albicans*	Pathogen
20	Tongue	*Lactobacillus rhamnosus*	Commensal
	Palate	*Streptococcus mitis*	Commensal
	Teeth	*Staphylococcus xylosus*	Commensal
	Periodontal	*Prevotella melaninogenica*	Pathogen
	Cheek	*Streptococcus oralis*	Pathogen
	Cheek	*Streptococcus mutans*	Pathogen
	Cheek	*Streptococcus salivarius*	Commensal
	Cheek	*Aerococcus urinae*	Pathogen
	Cheek	*Staphylococcus epidermidis*	Pathogen
	Saliva	*Lactobacillus acidophilus*	Commensal
21	Tongue	*Streptococcus salivarius*	Commensal
	Tongue	*Aerococcus urinae*	Pathogen
	Palate	*Streptococcus mitis*	Commensal
	Saliva	*Streptococcus salivarius*	Commensal

Relation between clinical history, hygiene habits and abundance of microorganisms.

Tongue brushing (p-value=0.0020), dental flossing (p-value=0.0070), use of prosthesis (p-value=0.0370), missing teeth (p-value=0.001), gingival bleeding (p-value=0.0004), teeth mobility (p-value=0.0002), treated caries (p-value=0.0060), untreated caries (p-value=0.0002) have significant statistical relation with the number of isolates retrieved, using the non-parametric Kruskal-Wallis test. Tongue brushing (p-value=0.0070), dental flossing (p-value=0.0010), use of prosthesis (p-value=0.0040), missing teeth (p-value=0.0010), gingival bleeding (p-value=0.0001), teeth mobility (p-value=0.0003), treated caries (p-value=0.0320), untreated caries (p-value=0.0003), have significant statistical relation with the number of pathogens.

Distribution of microorganisms within the oral cavity.

The similarity of the distribution of microbial isolates is displayed in Figure 1. The results indicate that the pathogen anaerobes *Prevotella sp*. and *Actinomyces naeslundii* have a similar distribution and are more associated to periodontal zone. Commensal *Lactobacillus*, yeasts (*Candida albicans*) and pathogenic *Staphylococci* are more associated to saliva. Species more associated to palate, cheek and tongue do not clearly separate from those labeled as general by occurring with similar frequency in more than two compartments. However, the pathogens *Leuconostoc spp* and *Enterococcus avium* were exclusively isolated from the palate; the pathogens *Streptococcus anginosus*, *Streptococcus termophilus* and *Streptococcus pneumoniae* were exclusively isolated from the cheek; the pathogens *Enterococcus faecalis*, Enterococcus faecium, *Staphylococcus chromogenes*, *Staphylococcus saprophyticus* and the commensals *Lactobacillus salivarius* and *Streptococcus uberis* were exclusively isolated from the tongue. The commensal *Staphylococcus xylosus* and pathogen *Streptococcus sanguinis*, more associated with teeth, have similar distributions. The pathogens *Candida albicans* and *Streptococcus oralis* associated with are also found in the teeth. The most numerous group is composed by species labeled as general because they could not be clearly associated with a single compartment. Some strains such as *Streptococcus mitis* and *Streptococcus salivarius*, were found in all the different zone of the oral cavity, except in the endodontics. *Streptococcus* dominate the strains with general distribution but the opportunistic pathogens *Aerococcus urinae*, *Gemella morbillorum* and *Staphylococcus epidermidis* are also found in this group.

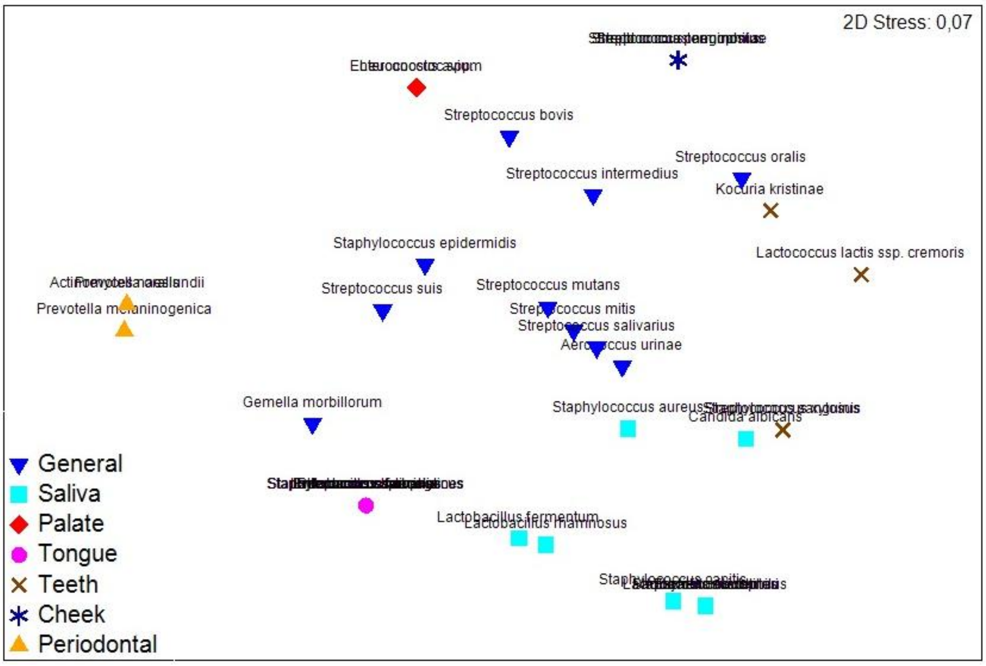

FIGURE 1 – MULTIDIMENSIONAL SCALING (MDS) BIDIMENSIONAL PLOT REPRESENTING THE RELATION BETWEEN MICROBIAL ISOLATES AND THEIR DISTRIBUTION. THE PREFERENTIAL ZONE OF OCCURRENCE IS IDENTIFIED BY THE SYMBOL BELOW THE SPECIES NAME. SPECIES OCCURRING IN IDENTICAL FREQUENCIES IN MORE THAN ONE ZONE WERE LABELED AS GENERAL.

The similarity between the microbial communities associated to the different micro-niches of the oral cavity is displayed in Figure 2. The periodontal zone and tongue are clearly distinct in terms of microbial colonization.

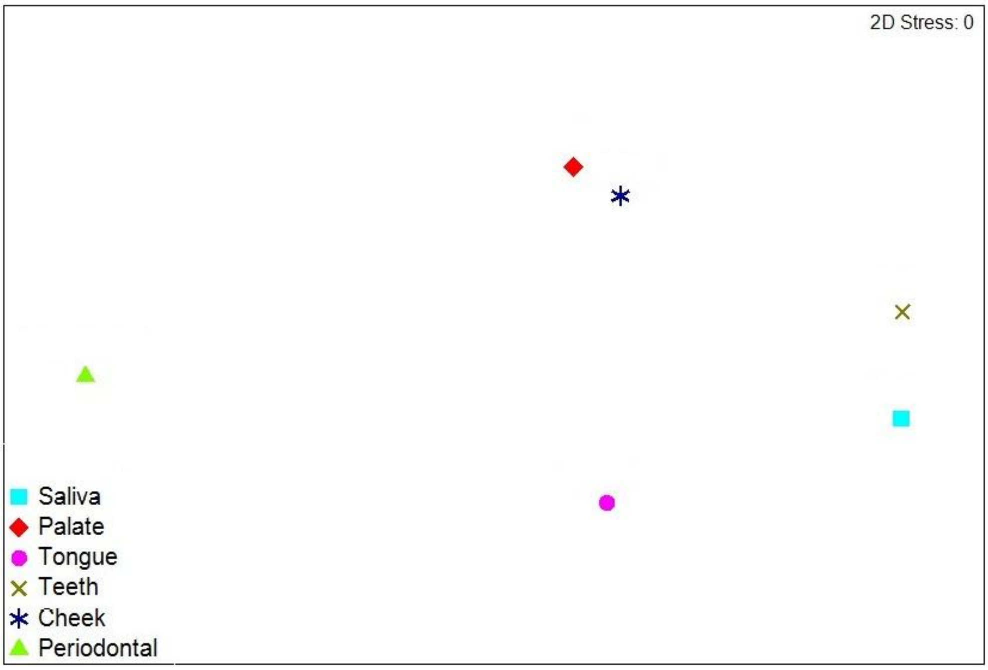

FIGURE 2 – MULTIDIMENSIONAL SCALING (MDS) BIDIMENSIONAL PLOT REPRESENTING THE SIMILARITY BETWEEN THE DIFFERENT COMPARTMENTS OF THE ORAL CAVITY IN TERMS OF THE COMPOSITION OF THE MICROFLORA ISOLATED FROM EACH COMPARTMENT.

The values of similarity within groups displayed in Table 3 indicate that palate, tongue and cheek have very stable communities, whereas the group labeled as general is the more heterogeneous; the high values of dissimilarity (close or equal to 100%) show a solid separation between the different groups confirming the above mentioned observation of the bidimensional MDS plot.

TABLE 3 – SIMPER VALUES OF SIMILARITY WITHIN ZONES (GREY CELLS), AND DISSIMILARITY BETWEEN ZONES (WHITE CELLS).

	Average Dissimilarity (%)						
Average Similarity (%)	*Periodontal*	*General*	*Saliva*	*Palate*	*Tongue*	*Teeth*	*Cheek*
Periodontal	**88.56**	89.70	100.00	100.00	100.00	100.00	100.00
General	89.70	**42.17**	79.79	66.65	75.11	71.77	70.58
Saliva	100.00	79.79	**65.93**	100.00	82.36	67.27	100.00
Palate	100.00	66.65	100.00	**100.00**	100.00	100.00	100.00
Tongue	100.00	75.11	82.36	100.00	**100.00**	100.00	100.00
Teeth	100.00	71.77	67.27	100.00	100.00	**63.44**	86.60
Cheek	100.00	70.58	100.00	100.00	100.00	86.60	**100.00**

ANOSIM value for species per zone of the mouth was global R=0.765, with a significance level of sample statistic of 0.1%, being close to 1 showing a statistical relevant separation between groups. Therefore, the displayed SIMPER values and the MDS 2D plots are statistical relevant.

DISCUSSION

This chapter intended to find relevant relations between clinical history events and hygiene habits and the incidence of particular commensal or pathogenic microbes, and to trace distribution profiles according to different mouth zones. Some hygiene habits were identified as having a direct influence in the colonization of the mouth by pathogenic and non-pathogenic microorganisms. Tongue brushing was significantly associated to the number of isolates and to the number of pathogens. The tongue has a surface with a particular texture and provides movement inside the mouth, being in physical contact with most of the other areas [1, 3]. So, tongue brushing has direct influence not only in pathogens colonization, as also influences all the microbial population dynamics and dissemination [1, 3]. Dental flossing was also statistically related to pathogen colonization. Flossing is a routine that allows reaching some critical micro-niches in human mouth, specifically in interdental space and, therefore, patients with no flossing habit are more likely to host pathogens [1]. Several medical history events and symptoms show a relation with microbial populations. As expected, non-treated caries and teeth mobility favors microbial colonization, as shown in their statistical relation with the total of isolates, and also with the number of pathogens. The use of prosthesis may be a cause for the colonization of pathogens, as the results suggest, probably as a consequence of the lack of proper hygiene. Prosthesis handling may also be a pathway of transmission of pathogens. Missing teeth is also statistically related with the abundance of pathogens, probably because it is also associated with the use of prosthesis. Several relations between different clinical events may be inferred taking into consideration their relation with microbial colonization. Poor oral hygiene and the consequent presence of pathogens can lead to teeth deterioration, exodontias and subsequent use of prosthesis which, ultimately enhance the risk of pathogen colonization. Gingival bleeding is commonly a symptom of infection and the results confirm a significant relation between this clinical event and the presence of teeth pathogens (*Streptococcus mutans* and *Prevotella oralis*, for example) [6].

The oral cavity is a complex ecosystem. However, the distribution and colonization by microorganisms is not random. The results point to niche specificities of microbial communities within the oral cavity. The periodontal community is notably separated by having typical species, namely anaerobic pathogens. Due to the particular micro-environment of this site, anaerobes are better adapted, in opposition to the other compartments of the mouth, unlikely to harbor anaerobes [23]. The saliva hosts a characteristic group of microaerophilic bacteria (*Lactobacilli*), possibly do to the concentration lower diffusion of oxygen [28]. The presence of yeasts (*Candida albicans*) also seems to be favored in the high humidity environment of the saliva [33]. Palate, tongue and cheek also demonstrate a relevant separation from the rest of the zones. The characteristic mucosal nature of these zones may influence the preferentially hosted microbes. Meanwhile, many bacteria are widely distributed in several zones of the mouth. The generalist group is mostly constituted for *Streptococcus*, possibly due the adaptability of these organisms to different environmental conditions.

The periodontal zone was the compartment more associated to relevant pathogens for oral health, while in mucosal zones and in the saliva fluid occurred more pathogens associated to systemic diseases (cancer, diabetes, endocarditis, among others) were isolated. This may be related to poor hygiene habits in relation to tongue brushing and dental flossing in the extent that a careful oral hygiene, not restricted to dental brushing, can help to prevent the colonization of some opportunistic pathogens.

SIMPER values show the average similarity percentage in each group, and also the dissimilarity values between groups. The group general referring to the community of species with similar abundance in several areas of the mouth have similarity of 42.17%, below 50%, reflecting the diversity of taxa and distribution present in this group, due to the specific characteristics of the oral cavity and richness in terms of microbial abundance. The other groups show high within group similarity. The high dissimilarity values between groups also reinforce the visible separation of the microbial communities through the different sites. Consequently it is possible to establish groups associated to different zones of the mouth with statistical relevance, and confirms that oral colonization is influenced by zones and organisms characteristics.

In summary, this chapter demonstrates statistical relations between hygiene habits and medical history events, and the number of isolates and pathogens, using clinical samples and information obtained from of dental clinic patients. This supports the relevance currently attributed to oral hygiene habits as major determinants of oral health, and also in the global health of the individual.

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CHAPTER 2 – EVALUATION OF THE BIOCIDAL ACTIVITY OF FUNCTIONALIZED OLIGOMERS

OVERVIEW

Oral microbes have clinical relevance and may be related to infections of the oral cavity and other organic systems. This section describes the evaluation of the biocidal activity of PMETOX-DDA [oligo(2-methyl-2-oxazoline) quaternized with N,N-dimethyldodecylamine] and LPEI [linear oligo(ethylenimine) hydrochloride] in the perspective of their future application as oral disinfectants. The synthesis of the polymers was previously conducted in the context of the research project PTDC/QUI/73939/2006 "ACTIVPOL - Development of poly-oxazolines and poly-aziridines with antimicrobial activity using supercritical technology". Methodologies of sampling and identification of oral microbes were based on clinical culture-dependent methods. MIC values were calculated to assess the efficiency of the studied poly(oxazoline)s as antimicrobial agents. This study provides the scientific basis to set poly(oxazoline)s are a valid alternative to conventional antibiotics, specifically when applied to microorganisms isolated from the oral cavity.

INTRODUCTION

Due to their structural and functional characteristics, namely chemical stability, reduced residual toxicity, nonvolatile properties, restricted permeation through the skin and versatility of effects as biocidal agents, the potential use of poly(oxazoline)s as antimicrobials against bacteria and fungi has been intensively studied in the past few years [1-6]. Even though the antimicrobial mechanisms of action of these polymers remain unknown, it is thought to be identical to that of poly(peptides) [4]. According to this hypothesis the bacteria surface attracts cationic antimicrobial peptides by electrostatic interaction between them and the anionic lipopolysaccharide (LPS) in outer membrane of gram-negative bacteria. In gram-positive bacteria the peptides are attracted by the negatively charged teichoic acids of the thick peptidoglycan layer [7, 8]. Both structural cell wall conformations are observable in Figure 1.

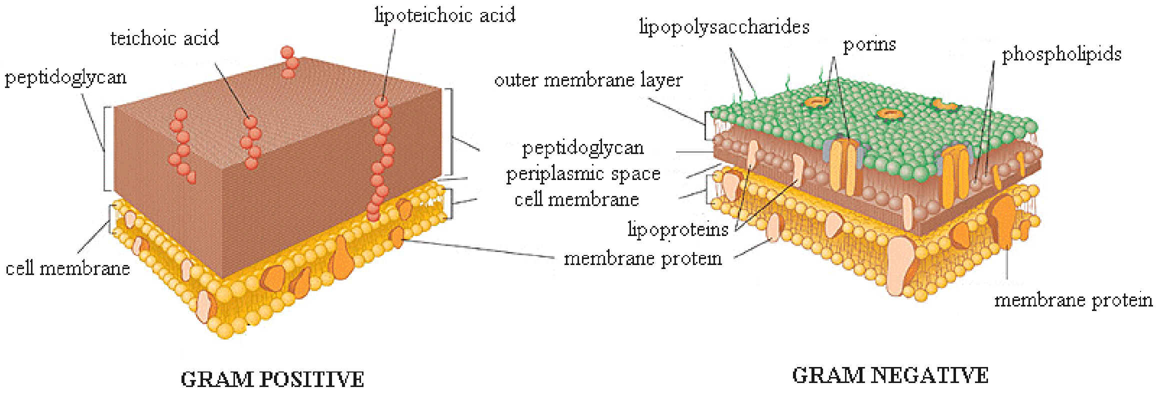

FIGURE 4 – MOLECULAR COMPOSITION OF GRAM POSITIVE AND GRAM-NEGATIVE BACTERIA, DISPLAYING MAIN DIFFERENCES BETWEEN BOTH: PEPTIDOGLYCAN THICKNESS AND THE PRESENCE OF OUTER MEMBRANE IN GRAM-NEGATIVE BACTERIA (ADAPTED FROM REF [9]).

Therefore, it is believed that antimicrobial peptides, and poly(oxazoline)s, present some degree of selectivity to negatively charged microbial cell envelopes in opposition to neutral mammalian cytoplasmic membranes. In a basic overview on the mechanism of action of antimicrobial peptides, it is hypothesized that they attach to the bacterial surface, cross the outer membrane of gram-negative cells, or the thick layer of gram-positive cells, by a self-promoted uptake to reach the anionic surface of the cytoplasmic membrane [8]. Then, peptides disrupt cell membrane by forming pores (barrel-stave model or toroidal-pore model) or by accumulating on the bilayer surface, covering it, dissolving the membrane in a detergent-like mode (carpet model) [7]. The two mechanisms are represented in Figure 2.

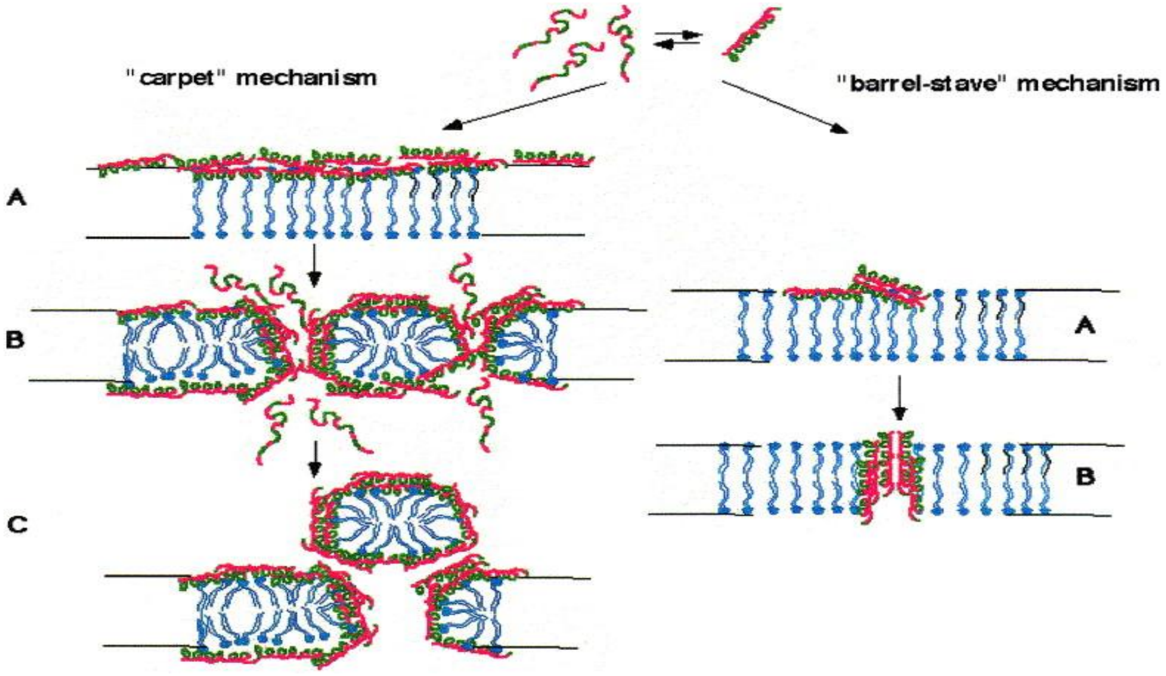

FIGURE 5 – MODEL OF MEMBRANE DISRUPTION BY THE CARPET MECHANISM (LEFT). THE ANTIMICROBIAL AGENT BINDS (A) AND ACCUMULATES IN THE MEMBRANE SURFACE, CONTINUED ACCUMULATION AND COVERING ("CARPETING") OF THE BILAYER (B) LEEDS TO A DETERGENT-LIKE DISINTEGRATION (C). MODEL OF TRANSMEMBRANE CHANNEL FORMATION BY THE BARREL STAVE MECHANISM (RIGHT). ANTIMICROBIAL AGENT ASSOCIATES TO THE MEMBRANE SURFACE (A) AND ACCUMULATES. ONCE A CRITICAL ANTIMICROBIAL AGENT/LIPID RATIO IS REACHED, AN INSERTION INTO THE MEMBRANE IS OBSERVED, WITH THE FORMATION OF BARREL-STAVE TYPE PORE (B) (ADAPTED FROM REF [10]).

According to the previously described models of interaction between antimicrobial peptides and the outer cell structures, development of resistance to these types of polymers would imply a complete remodeling of the membrane structure. These facts opens new perspectives for the therapeutic use of poly(oxazoline)s, as they are less likely to trigger the development of antimicrobial resistance, when compared to conventional antimicrobial agents [11].

The oral cavity has one of the richest and diverse microbial communities in human body. More than 500 species has been described in a human mouth [12]. However, 40%-60% of bacterial taxa found in the mouth have not yet been cultivated and properly identified, mainly because many of them are not cultivable in laboratory, and others, due to their metabolic and respiratory characteristics, are extremely difficult to maintain and preserve through conventional techniques [13-16]. Many clinical conditions have found directly related to oral infections [17, 18]. In the past few years, several studies have been conducted with the objective of demonstrating the role of oral microorganisms in pathologies such as endocarditis, cancer, respiratory infections and diabetes [19-22]. Because of its diversity in terms of microbial taxa, respiratory mechanisms and metabolic systems within a single microbial ecosystem, the oral microflora is ideal for testing new antimicrobial compounds. Therefore, in this study, the oral flora was chosen as model mixed bacterial assemblage to test effect of two new functionalized polymers (LPEI and PMETOX-DDA) obtained using supercritical carbon dioxide technology [23].

Considering the potential of functionalized polymers, an extensive testing program was conducted. For that, 103 wild strains of 37 different species, collected from 6 different areas of the mouth of 21 patients, were isolated, identified and tested in order to determine their MIC values, demonstrating the potential of the two synthesized polymers for application to clinical isolates.

MATERIALS AND METHODS

Isolation and identification of clinical strains

The clinical isolates were isolated and identified as described in Chapter 1.

Synthesis of polymers

The polymerizations of PMETOX-DDA [oligo(2-methyl-2-oxazoline) end-capped with N,N-dimethyldodecylamine] (Figure 6) and LPEI [linear oligo(ethylenimine) hydrochloride] (Figure 7) were carried out in a stainless steel reactor, using boron trifluoride etherate as the initiator [23].

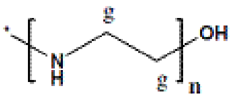

FIGURE 6 – SCHEMATIC REPRESENTATION OF LPEI (ADAPTED FROM [23]).

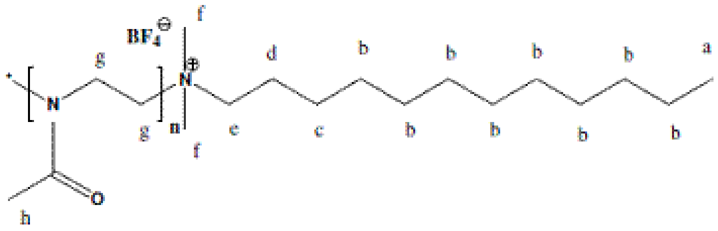

FIGURE 7 – SCHEMATIC REPRESENTATION OF PMETOX-DDA (ADAPTED FROM [23])

Determination of Minimum Inhibitory Concentration (MIC)

Stock cultures were recovered from Cryobank vials and cultured in CDC for anaerobic species, Blood Agar for aerobic species, MRS for microaerophilic species and Sabouraud (BD-Difco) for *Candida albicans*, under the adequate conditions of atmosphere and temperature. After 3 subcultures, a colony of each strain was transferred to 3 ml of Mueller-Hinton Broth (MHB) (BD – Difco) or to 3 mL of MHB with 5 % of synthesized horse blood for anaerobic strains. All strains were incubated at 37 °C until log phase of growth, assessed by optic density (OD) using a Densimat densitometer (bioMérieux) (approximately 24 hours for aerobic or facultative anaerobic microorganisms, 48-72 hours for anaerobes and microaerophilic microorganisms) and adjusted to 2.5×10^7 bacteria.mL^{-1} (according to McFarland units).

The MIC of the polymers was determined for each microorganism by turbidimetry. For the assay 96-well plates (Figure 8) were prepared as follow: 100 µl of MHB was placed in each well with a specific concentration of polymer. Sequential twofold dilutions were done, with MHB, to yield the desired concentrations of 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.098, 0.048, 0.024, 0.012 and 0.006 mg.mL^{-1}. Then, each well was inoculated with 5 µl of culture in order to have a final concentration of 5×10^6 cells per well. The microplates were incubated at 37 ºC during 24 h or 48 h-72 h for strict anaerobic and microaerophilic bacteria. Positive control (MHB inoculated with the respective tested microorganism), negative controls (MHB + polymer and MHB).

Escherichia coli AB1157 and *Staphylococcus aureus* NCTC8325-4 served as control organisms so that data could be compared to with results available in recent literature on in vitro susceptibility [23].

Determinations of OD 600 nm were conducted in an AMP Diagnostics automated microplate reader (model Platos R 496) after mechanical agitation of the plate. All the assays were repeated 3 times and each assay included duplicates.

Descriptive statistics and ANOVA were performed using the software XL-STAT (Microsoft).

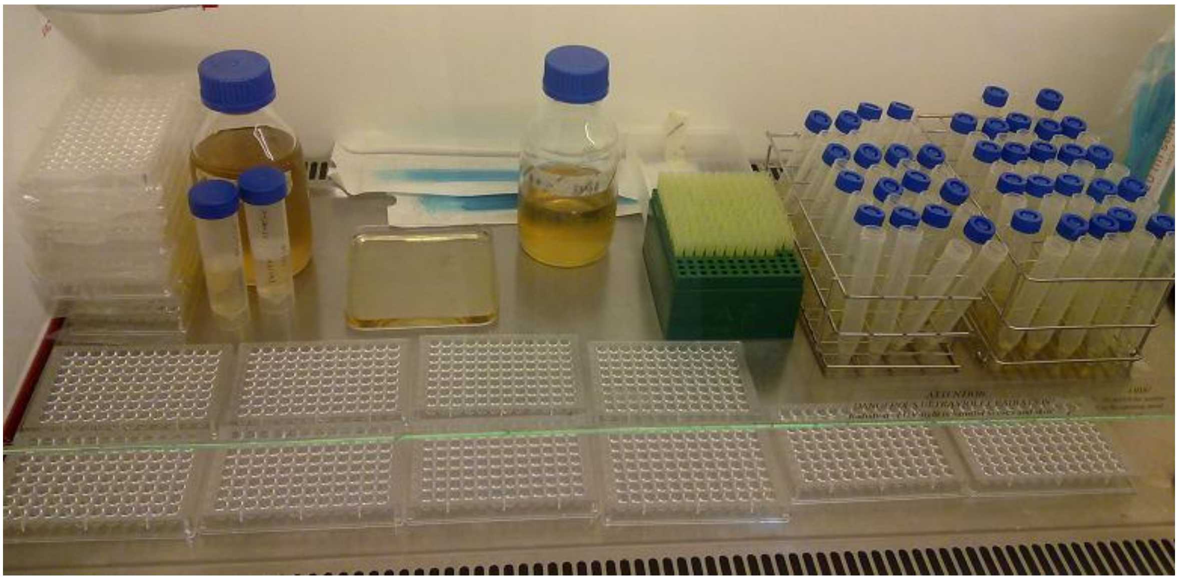

FIGURE 8 – DISPLAY OF 96-WELL PLATES, MHB CULTURE MEDIUM, ISOLATES AFTER 24H INCUBATION IN MHB AND DISSOLVED POLYMERS IN A LAMINAR FLOW CABINET.

RESULTS

A collection of 103 strains belonging to 37 different species was isolated and identified. The most frequent species were *Streptococcus salivarius* (n=13), *Aerococcus urinae* (n=10), *Staphylococcus aureus* (n=9) and *Streptococcus mitis* (n=9), showing a dominance of *Streptococcus* genus. Saliva was the biological product from which more isolates were obtained (n=28). Antimicrobial activity of PMETOX-DDA and LPEI was screened against all the clinical isolates (Table 1).

After statistical treatment of the raw data the average calculated MIC was 0.530 mg.mL^{-1} for LPEI and 0.723 mg.mL^{-1} for PMETOX-DDA. However the variance (0.466), standard deviation (0.683) and asymmetry values (Pearson=2.377; Fisher=2.413) associated to LPEI are higher, revealing a wider range of results and significant difference in the susceptibility of oral microorganisms to this polymer, in comparison to PMETOX-DDA (variance 0.337, standard deviation 0.581 and asymmetry values Pearson=1.572, Fisher=1.596). The results of the statistical analysis are represented in Figure 9.

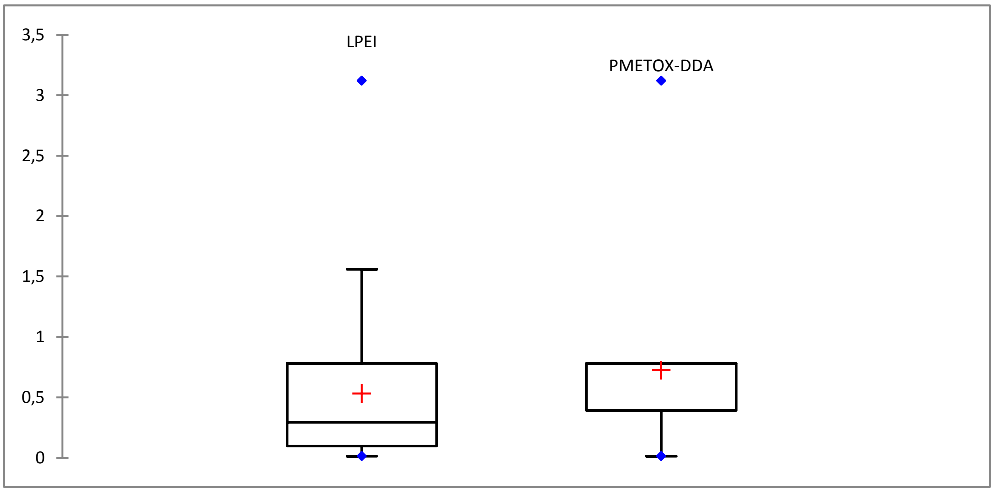

FIGURE 9 – BOXPLOT OF THE MIC OF LPEI AND PMETOX-DDA DETERMINED IN A SET OF 103 MICROBIAL STRAINS ISOLATED FROM HUMAN ORAL CAVITY. BLUE DOTS ARE THE EXTREME VALUES OBTAINED (MINIMUM=0.012 MG.ML^{-1}, MAXIMUM=3.12 MG.ML^{-1}, FOR BOTH POLYMERS), THE RED CROSS REPRESENTS THE MEAN, THE WHISKERS AND SIZE OF THE BOX REPRESENT THE QUARTILES AND DISPERSION VALUES.

Averages of MIC values vary between bacterial species and tested polymer (Figure 10). The clinically relevant species that presented higher resistance to LPEI than to PMETOX-DDA were*: Streptococcus oralis*, an important opportunistic pathogen and the most virulent species of the streptococci viridans group for being the producer of sialidase, an exo-glycosidase responsible for various diseases [24], *Aerococcus urinae*, potential causative agent of opportunistic endocarditis, *Enterococcus faecalis* and *Streptococcus anginosus*, also important potentially pathogens [7, 23]. Other species that were more resistant to LPEI than PMETOX-DDA are mostly commensals and common constituents of some probiotic foods. *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus pneumoniae* and *Streptococcus sanguinis* presented higher MIC values for PMETOX-DDA than for LPEI. These bacteria are well known for their potentially pathogenic nature in the oral cavity but are also directly or indirectly related to diseases in other organic systems in humans. Species with the lowest resistance (average MIC per species) to both polymers were *Lactobacilli* (< 0.1 mg.mL^{-1}), *Lactococcus lactis ssp. cremoris* (< 0.1 mg.mL^{-1}), *Leuconostoc sp*. (< 0.1 mg.mL^{-1}) and the anerobe pathogen *Actinomyces naeslundii* (< 0.1 mg.mL^{-1}).

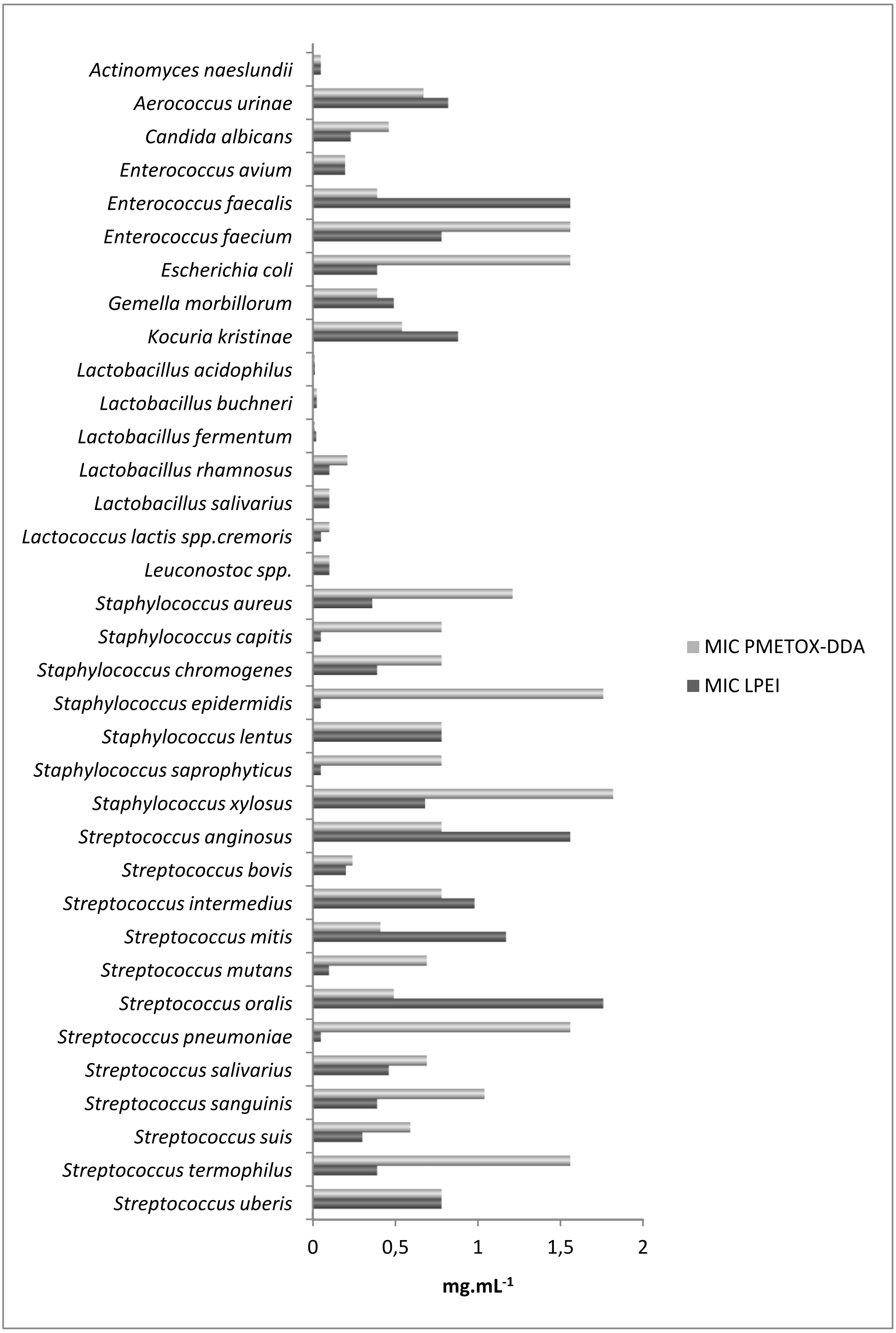

FIGURE 10 – VALUES OF AVERAGE MIC OF LPEI AND PMETOX-DDA (MG.ML^{-1}) DETERMINED FOR 37 SPECIES OF BACTERIA AND FUNGI ISOLATED FROM HUMAN ORAL CAVITY.

TABLE 1 – CHARACTERIZATION OF MICROBIAL ISOLATES IN TERMS OF ORAL COMPARTMENT, TYPE OF METABOLISM, PATHOGENICITY AND MIC VALUES FOR LPEI AND PMETOX-DDA.

Patient	Mouth Zone	Species	Metabolism	MIC LPEI mg.mL^{-1}	MIC PMETOX-DDA mg.mL^{-1}
1	Palate	*Streptococcus mitis*	Fac. Anaerobe	1.560	0.390
1	Cheek	*Streptococcus mitis*	Fac. Anaerobe	0.390	0.390
2	Palate	*Streptococcus intermedius*	Fac. Anaerobe	1.560	0.780
2	Teeth	*Streptococcus intermedius*	Fac. Anaerobe	0.390	0.780
2	Saliva	*Staphylococcus lentus*	Fac. Anaerobe	0.780	0.780
2	Saliva	*Candida albicans*	Fac. Anaerobe	0.390	0.390
3	Tongue	*Streptococcus uberis*	Fac. Anaerobe	0.780	0.780
3	Teeth	*Streptococcus sanguinis*	Fac. Anaerobe	0.195	1.560
3	Teeth	*Aerococcus urinae*	Fac. Anaerobe	0.780	0.780
3	Teeth	*Staphylococcus xylosus*	Fac. Anaerobe	0.098	0.780
4	Palate	*Streptococcus bovis*	Fac. Anaerobe	0.195	0.390
4	Saliva	*Streptococcus salivarius*	Fac. Anaerobe	0.390	0.780
5	Palate	*Streptococcus salivarius*	Fac. Anaerobe	0.780	0.780
5	Teeth	*Kocuria kristinae*	Fac. Anaerobe	0.780	0.780
5	Teeth	*Lactococcus lactis spp.cremoris*	Fac. Anaerobe	0.048	0.098
5	Cheek	*Aerococcus urinae*	Fac. Anaerobe	0.390	0.098
5	Cheek	*Streptococcus salivarius*	Fac. Anaerobe	0.390	0.780
5	Saliva	*Streptococcus mitis*	Fac. Anaerobe	3.120	0.195
5	Saliva	*Staphylococcus aureus*	Fac. Anaerobe	0.195	0.780
5	Saliva	*Staphylococcus xylosus*	Fac. Anaerobe	0.390	1.560
5	Saliva	*Escherichia coli*	Fac. Anaerobe	0.390	1.560
6	Teeth	*Kocuria kristinae*	Fac. Anaerobe	0.390	0.390
6	Saliva	*Staphylococcus aureus*	Fac. Anaerobe	0.390	1.560
6	Saliva	*Candida albicans*	Fac. Anaerobe	0.195	0.780
6	Saliva	*Lactobacillus rhamnosus*	Microaerophilic	0.048	0.390
7	Tongue	*Lactobacillus salivarius*	Microaerophilic	0.098	0.098
7	Teeth	*Candida albicans*	Fac. Anaerobe	0.195	0.390
7	Saliva	*Staphylococcus capitis*	Fac. Anaerobe	0.048	0.780
7	Saliva	*Streptococcus salivarius*	Fac. Anaerobe	0.390	0.780
7	Saliva	*Aerococcus urinae*	Fac. Anaerobe	0.780	0.780
7	Saliva	*Lactobacillus rhamnosus*	Microaerophilic	0.048	0.048
8	Tongue	*Streptococcus mitis*	Fac. Anaerobe	0.780	0.780
9	Tongue	*Staphylococcus saprophyticus*	Fac. Anaerobe	0.048	0.780
9	Teeth	*Streptococcus mitis*	Fac. Anaerobe	0.048	0.780
9	Teeth	*Aerococcus urinae*	Fac. Anaerobe	0.390	0.780
9	Teeth	*Streptococcus sanguinis*	Fac. Anaerobe	0.780	780
9	Periodontal	*Prevotella melaninogenica*	Strict Anaerobe	<0.006	<0.006
9	Saliva	*Staphylococcus capitis*	Fac. Anaerobe	0.048	0.780
10	Saliva	*Streptococcus sanguinis*	Fac. Anaerobe	0.195	0.780
11	Periodontal	*Staphylococcus epidermidis*	Fac. Anaerobe	0.048	0.780
11	Saliva	*Aerococcus urinae*	Fac. Anaerobe	0.048	0.195
12	Teeth	*Staphylococcus aureus*	Fac. Anaerobe	0.195	1.560
13	Periodontal	*Gemella morbillorum*	Fac. Anaerobe	0.780	0.390
14	Palate	*Streptococcus salivarius*	Fac. Anaerobe	1.560	0.780
14	Saliva	*Candida albicans*	Fac. Anaerobe	0.195	0.390
15	Tongue	*Streptococcus salivarius*	Fac. Anaerobe	0.390	0.390
15	Teeth	*Streptococcus salivarius*	Fac. Anaerobe	0.390	1.560
15	Teeth	*Staphylococcus aureus*	Fac. Anaerobe	0.195	1.560
15	Periodontal	*Prevotella oralis*	Strict Anaerobe	<0.006	<0.006
15	Cheek	*Streptococcus mutans*	Fac. Anaerobe	0.098	0.048

15	Saliva	*Staphylococcus aureus*	Fac. Anaerobe	0.195	1.560
15	Saliva	*Candida albicans*	Fac. Anaerobe	0.195	0.390
16	Tongue	*Gemella morbillorum*	Fac. Anaerobe	0.195	0.390
16	Tongue	*Lactobacillus fermentum*	Microaerophilic	0.024	0.012
16	Cheek	*Streptococcus anginosus*	Fac. Anaerobe	1.560	0.780
16	Cheek	*Kocuria kristinae*	Fac. Anaerobe	1.560	0.780
16	Saliva	*Staphylococcus aureus*	Fac. Anaerobe	0.048	0.780
17	Tongue	*Aerococcus urinae*	Fac. Anaerobe	0.780	0.780
17	Tongue	*Staphylococcus epidermidis*	Fac. Anaerobe	0.048	1.560
17	Palate	*Streptococcus suis*	Fac. Anaerobe	0.390	0.390
17	Palate	*Streptococcus salivarius*	Fac. Anaerobe	0.780	0.195
17	Teeth	*Streptococcus salivarius*	Fac. Anaerobe	0.195	0.195
17	Teeth	*Staphylococcus aureus*	Fac. Anaerobe	0.048	0.780
17	Cheek	*Streptococcus mitis*	Fac. Anaerobe	3.120	0.780
17	Cheek	*Streptococcus pneumoniae*	Fac. Anaerobe	0.048	1.560
17	Saliva	*Lactobacillus buchneri*	Microaerophilic	0.024	0.024
18	Tongue	*Streptococcus suis*	Fac. Anaerobe	0.195	0.780
18	Palate	*Streptococcus salivarius*	Fac. Anaerobe	0.390	0.780
18	Teeth	*Kocuria kristinae*	Fac. Anaerobe	0.780	0.195
18	Teeth	*Aerococcus urinae*	Fac. Anaerobe	0.780	0.195
18	Cheek	*Streptococcus bovis*	Fac. Anaerobe	0.195	0.098
18	Saliva	*Lactobacillus fermentum*	Microaerophilic	0.012	0.012
19	Tongue	*Enterococcus faecium*	Fac. Anaerobe	0.780	1.560
19	Tongue	*Staphylococcus chromogenes*	Fac. Anaerobe	0.390	0.780
19	Tongue	*Staphylococcus aureus*	Fac. Anaerobe	1.560	1.560
19	Tongue	*Enterococcus faecalis*	Fac. Anaerobe	1.560	0.390
19	Palate	*Streptococcus mitis*	Fac. Anaerobe	1.560	0.390
19	Palate	*Leuconostoc spp.*	Fac. Anaerobe	0.098	0.098
19	Palate	*Streptococcus mutans*	Fac. Anaerobe	0.098	0.390
19	Palate	*Enterococcus avium*	Fac. Anaerobe	0.195	0.195
19	Palate	*Staphylococcus epidermidis*	Fac. Anaerobe	0.048	1.560
19	Teeth	*Streptococcus oralis*	Fac. Anaerobe	3120	0.780
19	Periodontal	*Actinomyces naeslundii*	Strict Anaerobe	0.048	0.048
19	Cheek	*Aerococcus urinae*	Fac. Anaerobe	3.120	0.780
19	Cheek	*Streptococcus mitis*	Fac. Anaerobe	0.195	0.195
19	Cheek	*Streptococcus termophilus*	Fac. Anaerobe	0.390	1.560
19	Saliva	*Streptococcus mutans*	Fac. Anaerobe	0.098	0.780
19	Saliva	*Staphylococcus aureus*	Fac. Anaerobe	0.390	0.780
19	Saliva	*Candida albicans*	Fac. Anaerobe	0.195	0.390
20	Tongue	*Lactobacillus rhamnosus*	Microaerophilic	0.195	0.195
20	Palate	*Streptococcus mitis*	Fac. Anaerobe	0.195	0.098
20	Teeth	*Staphylococcus xylosus*	Fac. Anaerobe	1.560	3.120
20	Periodontal	*Prevotella melaninogenica*	Strict Anaerobe	<0.006	<0.006
20	Cheek	*Streptococcus oralis*	Fac. Anaerobe	0.390	0.195
20	Cheek	*Streptococcus mutans*	Fac. Anaerobe	0.098	1.560
20	Cheek	*Streptococcus salivarius*	Fac. Anaerobe	0.098	0.780
20	Cheek	*Aerococcus urinae*	Fac. Anaerobe	0.390	0.780
20	Cheek	*Staphylococcus epidermidis*	Fac. Anaerobe	0.048	3.120
20	Saliva	*Lactobacillus acidophilus*	Microaerophilic	0.012	0.012
21	Tongue	*Streptococcus salivarius*	Fac. Anaerobe	0.048	0.390
21	Tongue	*Aerococcus urinae*	Fac. Anaerobe	0.780	1.560
21	Palate	*Streptococcus mitis*	Fac. Anaerobe	0.780	0.098
21	Saliva	*Streptococcus salivarius*	Fac. Anaerobe	0.195	0.780

DISCUSSION

The main goal of this chapter was to evaluate the efficiency of PMETOX-DDA and LPEI against wild microbial strains. For that, MIC values were determined for a set of 103 clinical isolates representing bacteria and yeasts of the human oral cavity. The tested compounds showed to be effective as antimicrobials, with no resistance obtained for all tested isolates.

MIC values for the antimicrobial polymers tested in this study are higher than that of conventional antimicrobials [25]. However poly(oxazoline)s show selectivity to bacterial cells, due to their negative charged nature, and are unlikely to cause collateral damage on tissues of the oral cavity. These antimicrobial polymers are also effective when against fungi, which can be related to negatively charged cell envelope of yeasts [26].

The MIC values were also used to compare the efficiency of inactivation of clinical isolates with these poly(oxazoline)s, with results of a previous work with the reference strains *E. coli* AB1157 and *S. aureus* NCTC8325-4. According to ANOVA analysis, the values obtained in the present study are not significantly different from those obtained by Correia *et al* [23]. In this work, MIC values do not present significant statistical differences in terms of general mean, when comparing the values corresponding to *E. coli* and *S. aureus* reference strains with clinical isolates, for both compounds.

Microorganisms present a high degree of genetic variability and that can explain some small variability in the thickness of the cell envelope and, therefore, in the susceptibility to poly(oxazoline)s between species [27]. The mechanism of action currently hypothesized for poly(oxazoline)s involves interaction with cell wall and cell membrane. Therefore, the susceptibility may vary with small differences in the thickness of cell envelope. This variability may influence the time of action (inhibition rate), but not the final inhibition factor, which was 100% in all tested strains.

Tested individually, PMETOX-DDA and LPEI showed low MIC values when compared to other antimicrobial polymers [23]. Even though MIC values PMETOX-DDA are more centered and statistically consistent, LPEI was in general, more effective than PMETOX-DDA. The complementarity between the two compounds in terms of range and average values of MIC per species may indicate that a combined formulation could significantly enhance antimicrobial efficiency.

Although further studies are required (currently being performed by our team), these results suggest that there are good perspectives for the application of PMETOX-DDA and LPEI to the field of antimicrobial drug development, and that these compounds may be used in antiplaque oral care formulations to inhibit the development of the dental plaque.

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CONCLUDING REMARKS

Beyond the clinical relevance of oral cavity, there is a scientific interest in this particular ecosystem due to its specific characteristics. Mouth has one of the most abundant and diverse microbial communities in human body, thereby, the performed study revealed to be rather important. The distribution of microorganisms in oral cavity showed to be influenced by several factors, being the action of the saliva and the tongue the most relevant. Their action helps to explain the existence of many microbes with a general distribution in the mouth. The patterns of distribution of microorganism are characterized by a higher frequency of anaerobic pathogens in the endodontic zone, and by the presence of systemic pathogens non-related with oral diseases in mucous zones, especially on the cheek, palate and tongue. The anaerobic microorganisms from the endodontic hold a prominent position in terms of oral disease. However, some well known aerobic and facultative anaerobic pathogens were isolated from the cheek, saliva, tongue and teeth.

Functionalized polymers, specifically PMETOX-DDA and LPEI, proved to be efficient in their antimicrobial action. Due to their currently accepted mechanism of action, these new antimicrobials are seen as promising complement, or even alternative, to conventional antibiotics. By being less prone to the development of resistance, there is the hope that the combat to the scourge of infectious diseases may be a war with victory for human been. Their specific action in oral microorganisms is an important advance in clinical treatment, not only of oral infections, but also of the related diseases that are often reported. Therefore, when comparing the demonstrated efficiency and broad range of antimicrobial polymers, their mechanism of action and low risk of developing resistant microbial communities, the benefits seem extremely promising. Additionally, the closely null toxicity was attested by recently performed tests. These tests reported that the first small rashes and lesions appear to start with concentrations around 10 mg.mL-1 (unpublished data). Since the maximum obtained MIC values were 3.12 mg.mL-1, these results suggest that is possible to use these compounds as a preventive application, or in critical situations use these compounds in aggressive therapeutics using higher concentrations, without side effects to the patient.

In future studies, it will be important to better study the specific action of polymers in anaerobes from endodontic zone, as well as their mechanism of action through the determination of killing curves and structural tests. An enlargement of the battery of tested microorganisms could, also, provide better understanding of the efficiency of the tested compounds. An approach using molecular methods and population screening and posterior susceptibility tests could also be extremely useful in the continuation of the study of these polymers. Molecular methods may also be useful in the study of distribution of microorganisms, by sampling from different zones and then performing population studies.

The ecological approach to the distribution of microorganisms in the oral cavity can provide the knowledge to the implementation of preventive practices that, combined with the antiseptic action of the tested functionalized polymers, may significantly contribute to an improvement in oral health.

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APPENDIX SECTION

APPENDIX INDEX

INSTITUTO SUPERIOR DE CIÊNCIAS DA SAÚDE
EGAS MONIZ

Consentimento Informado

Código | IMP.EM.EI.15_00

Exmo. (a) Sr. (a),

No âmbito da tarefa 'ActivPOL – Desenvolvimento de poli-oxazolinas e poli-aziridinas com actividade antimicrobiana utilizando tecnologia supercrítica' (PTDC/QUI/73939/2006), pretende-se recolher diferentes amostras (saliva, superfície da língua, placa dentária) da boca, por técnicos de saúde credenciados, para posterior isolamento de microrganismos que servirão para determinar a eficácia biocida de compostos sintetizados em tarefas anteriores do projecto mencionado.

Neste estudo não será efectuado qualquer tipo de tratamento ou diagnóstico, os dados recolhidos destinam-se apenas para um estudo estatístico.

Todas as informações recolhidas neste estudo serão apenas utilizadas na elaboração desta investigação e serão guardadas e processadas num computador, sendo confidenciais, não o(a) identificando individualmente.

O estudo final será publicado na biblioteca do ISCSEM, da Universidade de Aveiro e em revistas científicas, mas o paciente não será identificado em qualquer publicação ou relatório.

Tomei conhecimento que me foram prestadas todas as informações relacionadas com os objectivos e métodos do estudo, tendo sido esclarecido(a) em todas as minhas dúvidas e questões. Além disso fui informado(a) que sou livre de aceitar ou recusar participar neste estudo.

Eu, ____ aceito participar neste estudo e AUTORIZO[1] a recolha de amostras da minha boca para isolamento de microrganismos, o armazenamento da minha informação, a transferência e publicação dos meus dados.

Assinatura do investigador:____

Assinatura da testemunha:____

Monte de Caparica, ______ de ___________________ de 201__

---

(1) Caso o doente seja menor, devem assinar os pais ou o encarregado de educação.

EGAS MONIZ

Clínica Universitária

História Clínica

Agradecemos que preencha este questionário. A informação fornecida destina-se a proporcionar-lhe o melhor tratamento e é *confidencial*.
Sempre que, no futuro, houver alteração na sua história clínica, deve informar o seu médico assistente.

Responda, por favor, a todas as questões ou coloque um círculo no local correcto.

ANTECEDENTES PESSOAIS

Esteve internado em Hospital no último ano? SIM NÃO
*Se SIM, qual o nome e telefone do Médico assistente*________________________ Tel.: ____________

Está em tratamento médico? SIM NÃO
Se SIM, qual é a doença? ________________________

Tomou regularmente algum medicamento durante o último ano? SIM NÃO
Se SIM qual? ____
____

Tem alergia a algum medicamento? SIM NÃO
Se SIM qual? ________________________
Toma a pílula? SIM NÃO

Alguma vez teve hemorragias excessivas exigindo tratamento? SIM NÃO

Coloque um círculo nas doenças que tem ou teve:

Artrite
Candidíase oral ("sapinhos")
Doença cardíaca congénita
Doença da tiróide
Doença venérea
Enfarte miocárdio
Febre reumática
Gânglios aumentados de volume
Glaucoma

Úlcera gástrica/duodenal
Leucemia
Diabetes
Endocardite infecciosa
Infecção pelo vírus da Sida (HIV)
Anemia
Asma
Cancro
Hipertensão arterial

Hepatite viral (tipo____)
Doença valvular cardíaca
Prótese valvular cardíaca
Tratamento psiquiátrico
Tuberculose
Alergias
Tosse persistente
Portador de Pacemaker
Epilepsia

Alguma vez efectuou tratamento com radiação (Radioterapia)? SIM NÃO

(Para pessoas do sexo feminino) Está grávida? SIM NÃO

Tem alguma doença importante não mencionada acima? SIM NÃO
Se SIM qual? ________________________

HÁBITOS

Se é fumador(a), quantos cigarros fuma por dia? ____________

Costuma ingerir bebidas alcoólicas? SIM NÃO
Em que circunstâncias? ________________

É consumidor(a) de estupefacientes? SIM NÃO

HIGIENE ORAL

Quantas vezes escova os dentes ao dia?____________________
Qual o tipo de dentífrico?_____________________________
Escovagem da língua? ___ Sim ___ Não
Costuma usar fio ou escovilhão dentário? SIM NÃO
[illegible]

Costuma usar colutório? SIM NÃO
Se SIM, de que tipo?_____________________________
Utiliza suplemento c/ fluor? ___ Sim ___ Não

E portador(a) de Prótese Dentária? SIM NÃO
Se SIM, de que tipo?_____________________________
Há quanto tempo? _____________________________

Já fez algum tratamento para correcção dentária? SIM NÃO

Observações:____

ANTECEDENTES FAMILIARES

Ultima consulta de Medicina Dentária?____________________

Sabe o que é uma Clínica Universitária? SIM NÃO

Porque escolheu esta Clínica?____

Autorizo a utilização dos meus dados clínicos para fins científicos? SIM NÃO

Foi enviado(a) por quem? ____

Se está a preencher a ficha por outra pessoa, qual o grau de parentesco? ____________________

Declaro que as informações prestadas são verdadeiras

Assinatura: ____

EXAME OBJECTIVO

EXTRAORAL

Configuração crâniofacial - Tipo________________________________

Assimetrias ou alterações faciais? SIM NÃO
Localização?________________________________
De que tipo?________________________________
____

Dimensão vertical ____________________

Limitação dos movimentos mandibulares? Não Esquerda Direita
Presença sons articulares Não Esquerda Direita
Sintomatologia dolorosa da ATM? Não Esquerda Direita

Outras observações________________________________
____
____

INTRAORAL

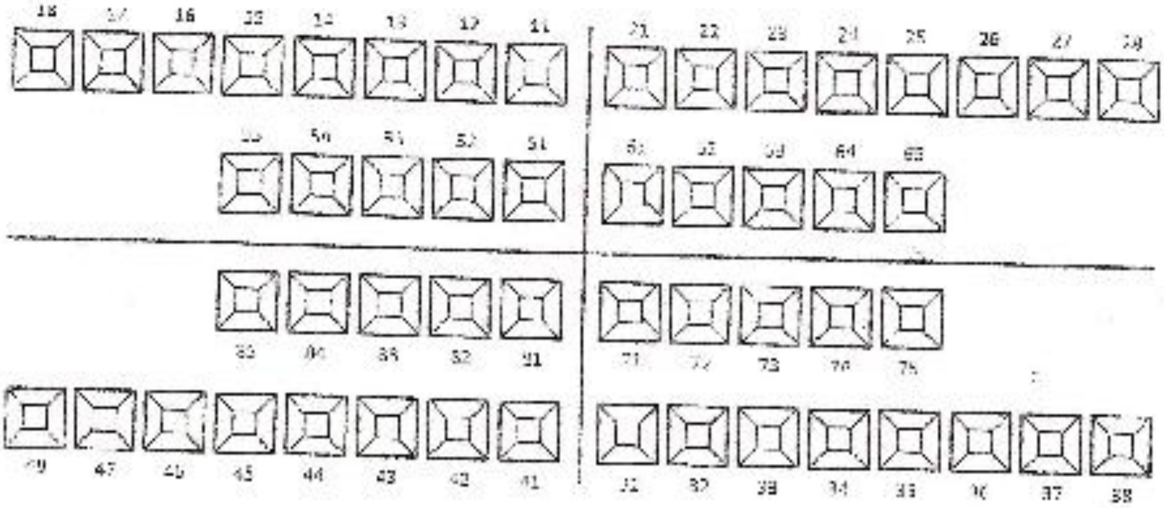
Alterações dos tecidos moles? SIM NÃO
Localização?________________________________
De que tipo?________________________________
____

Hemorragia gengival? SIM NÃO
Mobilidade Dentária? SIM NÃO

Alterações das estruturas dentárias? SIM NÃO
De que tipo?________________________________

Outras observações________________________________
____
____

FICHA DENTÁRIA INTERNACIONAL



DADOS PESSOAIS

PACIENTE Nº: ____ IDADE: ____ SEXO: ____

PROFISSÃO: ________________________________

NÍVEL DE ESCOLARIDADE: ____

HÁBITOS ALIMENTARES E HIGIÉNICOS

ONICOFAGIA (RÓI AS UNHAS?): SIM___ NÃO___

INGESTÃO DE DOCES: NÃO INGERE ____ NÃO TODOS OS DIAS ____

1XDIA: ____ 2XDIA: ____ 3 OU + DIA: ____

CONSIDERA TER UMA ALIMENTAÇÃO CUIDADA: SIM ____ NÃO ____

ESCOVAGEM DA LÍNGUA: SIM ____ NÃO ____

PARTILHA ESCOVA DE DENTES: SIM ____ NÃO ____

UTILIZA SUPLEMENTOS COM FLÚOR: SIM ____ NÃO ____

Nota: Os dados recolhidos de pacientes ou voluntários são anónimos e a sua utilização limitar-se-á a fins estatísticos, no âmbito do projecto PTDC/QUI/73939/2006 "ActivPOL- Desenvolvimento de poli-oxazolinas e poli-aziridinas com actividade antimicrobiana utilizando tecnologia supercrítica" e da tese de Mestrado de Celso Martins (Departamento de Biologia da Universidade de Aveiro).

Microbial Ecology

Relations between oral hygiene, medical history and composition of microbial communities of the human mouth

--Manuscript Draft--

Manuscript Number:	
Full Title:	Relations between oral hygiene, medical history and composition of microbial communities of the human mouth
Article Type:	Original Article
Corresponding Author:	Celso Martins PORTUGAL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Celso Martins
First Author Secondary Information:	
Order of Authors:	Celso Martins
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Abstract:	Abstract The purpose of this study was to analyze the influence of medical history and oral hygiene habits on the composition of oral microbial communities, and to trace incidence profiles of species in 6 zones of the mouth, taking into account their pathogenicity. Oral samples were collected from 21 patients, and bacterial strains were isolated and identified with conventional culture techniques. Assessment of medical history events and symptoms identification were performed by medical dentists and dentistry students. Statistical studies were performed based on descriptive statistics methods, correlation coefficients and multivariate ecological statistic methods, in order to obtain realistic distribution profiles and models using XL-STAT and PRIMER 6. A set of 103 strains, representing 37 different species was obtained. Statistically significant results of Kruskal-Wallis non-parametric test were obtained (p-value<0.05) for some hygiene habits and medical history events. Similarity of Percentages (SIMPER) analysis values of dissimilarity between groups were statistically relevant, as well as the Analysis of Similarities (ANOSIM) analysis (global R=0.765), allowing to infer the possibility to distinguish different zones according to the microbial distribution visible in Multidimensional Scaling (MDS) charts. Distribution profiles for the different isolates could be established and it was possible to find significant relations between microbial species and particular compartments within the oral cavity. The endodontic zone stands from the rest for its unique characteristics, hosting anaerobe oral pathogens, while most of systemic pathologies related microbes are mostly hosted by mucosal areas.
Suggested Reviewers:	

Evaluation of the biocidal activity of functionalized oligomers

Journal:	*Journal of Antimicrobial Chemotherapy*
Manuscript ID:	JAC-2012-0894
Manuscript Type:	Original Article
Date Submitted by the Author:	09-Jul-2012
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Keywords:	Antimicrobials, Oral microbiology, MIC, Cell wall